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| FORM PTO-1390 (Modified)<br>(REV 11-98)  |  | U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE |  | ATTORNEY'S DOCKET NUMBER<br><b>4001-0003</b>                                 |
| TRANSMITTAL LETTER TO THE UNITED STATES<br>DESIGNATED/ELECTED OFFICE (DO/EO/US)<br>CONCERNING A FILING UNDER 35 U.S.C. 371   |  |   |  | U.S. APPLICATION NO (IF KNOWN, SEE 37 CFR<br><b>To Be Assigned 09/720979</b> |
| INTERNATIONAL APPLICATION NO.<br><b>PCT/JP99/03552</b>   | INTERNATIONAL FILING DATE<br><b>01-July-1999</b> | PRIORITY DATE CLAIMED<br><b>03-July-1998</b>            |  |  |
| TITLE OF INVENTION<br><b>Negative-Sense RNA Virus Vector for Nerve Cell</b>  |  |   |  |  |
| APPLICANT(S) FOR DO/EO/US<br><b>FUKUMURA, et al.</b>   |  |   |  |  |
| Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:  |  |   |  |  |
| <p>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).</p> <p>4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371 (c) (2))<br/>       a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).<br/>       b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.<br/>       c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</p> <p>7. <input checked="" type="checkbox"/> A copy of the International Search Report (PCT/ISA/210).</p> <p>8. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))<br/>       a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).<br/>       b. <input type="checkbox"/> have been transmitted by the International Bureau.<br/>       c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.<br/>       d. <input type="checkbox"/> have not been made and will not be made.</p> <p>9. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>10. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).</p> <p>11. <input checked="" type="checkbox"/> A copy of the International Preliminary Examination Report (PCT/IPEA/409).</p> <p>12. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).</p> |  |   |  |  |
| <p><b>Items 13 to 20 below concern document(s) or information included:</b></p> <p>13. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>14. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>15. <input type="checkbox"/> A <b>FIRST</b> preliminary amendment.</p> <p>16. <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.</p> <p>17. <input type="checkbox"/> A substitute specification.</p> <p>18. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>19. <input type="checkbox"/> Certificate of Mailing by Express Mail</p> <p>20. <input checked="" type="checkbox"/> Other items or information:<br/>       1. Copy of the International Publication;<br/>       2. Copy of the PCT Request Form;<br/>       3. Copy of PCT/IPEA/416;<br/>       4. Copy of PCT/IPEA/409;<br/>       5. Copy of PCT/IPEA/408;<br/>       6. Copies of all papers received from WIPO (including PCT/IB/332, PCT/IB/308, PCT/IB/301, PCT/IB/304, and PCT/IB/345).<br/>       7. Copy of Demand for International Preliminary Examination; and<br/>       8. Copy of Written Opinion.</p>   |  |   |  |  |

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| U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR<br>To Be Assigned) <b>02/720979</b> | INTERNATIONAL APPLICATION NO.<br><b>PCT/JP99/03552</b> | ATTORNEY'S DOCKET NUMBER<br><b>4001-0003</b> |
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21. The following fees are submitted:

**BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :**

|   |          |
|---|----------|
| <input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... | \$970.00 |
| <input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but Internation Search Report prepared by the EPO or JPO .....   | \$840.00 |
| <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO .....   | \$690.00 |
| <input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) .....  | \$670.00 |
| <input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) .....  | \$96.00  |

**CALCULATIONS PTO USE ONLY**

**ENTER APPROPRIATE BASIC FEE AMOUNT =**

**\$840.00**

Surcharge of **\$130.00** for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492 (e)).  20  30 **\$0.00**

| CLAIMS             | NUMBER FILED | NUMBER EXTRA | RATE      |               |
|--------------------|--------------|--------------|-----------|---------------|
| Total claims       | 15 - 20 =    | 0            | x \$18.00 | <b>\$0.00</b> |
| Independent claims | 3 - 3 =      | 0            | x \$78.00 | <b>\$0.00</b> |

Multiple Dependent Claims (check if applicable).

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**TOTAL OF ABOVE CALCULATIONS =** **\$840.00**

Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable).  **\$420.00**

**SUBTOTAL =** **\$420.00**

Processing fee of **\$130.00** for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492 (f)).  20  30 + **\$0.00**

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Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).  **\$0.00**

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Please charge my Deposit Account No. in the amount of to cover the above fees. A duplicate copy of this sheet is enclosed.

The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **50-0622** A duplicate copy of this sheet is enclosed.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

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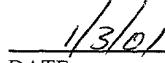
  
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DATE

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09/720979  
528 Rec'd PCT/PTO 03 JAN 2001

## DESCRIPTION

### NEGATIVE- SENSE RNA VIRUS VECTOR FOR NERVE CELL

#### 5    Technical Field

The present invention relates to a method of transferring a gene for gene therapy of nerve cells using a virus vector, more specifically, a negative-sense RNA virus vector.

#### Background Art

10       It is an extremely important object in the gene therapy for humans and animals to develop a system whereby a gene is transferred into target organs and target cells with a high efficiency. Methods for transferring a gene include the calcium phosphate method, DEAE-dextran method, cationic liposome method, electroporation method, etc., and especially methods for transferring a gene *in vivo* include a method using virus or liposome, or a direct transfer method. Among them, the gene transfer performed using "a virus vector" obtained by recombination of viral gene is extremely useful for the transfer of a gene into cells, for example, for gene therapy because of easy transfer procedure and its high transfer efficiency.

15       Virus vectors commonly used at present in gene therapy include retrovirus vector, herpes simplex virus (HSV) vector, adenovirus vector, and adeno-associated virus (AAV) vector, etc. In particular, along with the recent progress in analysis of brain functions using MRI and PET, there has been an increased demand for vectors capable of efficiently infecting non-dividing nerve cells and mediating a high level transgene expression in the infected cells. Therefore, adenoviral vector, herpes simplex viral vector, AAV, HIV, etc. have received considerable attention.

20       Although HSV has been reported to be capable of transferring a gene into ganglions in the peripheral nervous system, a problem remains on the amount of its expression (Gene Therapy, 1995, 2: 209-217). HIV infection of nerve cells has also been confirmed (Nature Biotechnology, 1997, 15: 871-875). Since the chromosomal position into which the HIV genome is inserted is hardly predictable, there are possibilities of damaging a normal gene, activating a cancer gene, and inducing excessive or suppressed expression of a desired gene.

25       AAV has been used for the brain treatment in Parkinson's disease (Exp. Neurol., 1997, 144: 147-156) and mucopolysaccharidosis type VII (ASGT meeting, 1998, Abstract No. 692). However, there have been reported an incomplete transfer of the introduced gene into the substantia nigra in Parkinson's disease and its insufficient expression in the brain in mucopolysaccharidosis type VII.

30       Adenovirus has been most commonly used at present, and reported to be capable of transferring a gene into the pyramidal cell layer of hippocampus (Nature Medicine, 1997, 3: 997-

1004). However, adenovirus has drawbacks, such as cytotoxicity and high immunogenicity.

On the other hand, since negative-sense RNA viruses, such as Sendai virus (hereinafter abbreviated as SeV), are not integrated into chromosomes, they do not activate cancer genes. Furthermore, since SeV is an RNA virus, it has advantages, such as protein expression in short time after infection and an extremely higher level expression of the transgene product compared with Adenovirus.

#### Disclosure of the Invention

It is an objective of this invention to provide a method for transferring nucleic acid using a negative-sense RNA viral vector. This method is useful for gene therapy of nerve cells, etc.

The present inventors first prepared recombinant viruses carrying various foreign genes, using SeV, a typical negative-sense RNA virus and useful as a vector for gene therapy because of its safety and convenience. Subsequently, these recombinants were used to transfer the foreign genes into nerve cells, brain tissues, etc. As a result, the inventors found that the use of these recombinants enabled an efficient transfer of foreign genes into nerve cells and brain tissues. Furthermore, they found that the use of viral vectors of this invention led to high level expression of foreign genes introduced.

In addition, viral vectors of this invention transferred into the brain exhibited the limited proliferation. In other words, the expression of the vectors was reduced after a certain period of foreign gene expression. Furthermore, the gene therapy using a viral vector of this invention was applied to the brain of a  $\beta$ -glucuronidase-deficient mouse, which improved the symptoms of said mouse. Thus, the present inventors discovered that the viral vectors prepared could efficiently function in gene therapy of neuropathy where the therapy requires regulation of transgene expression.

The intraventricular administration of a viral vector of this invention carrying an FGF gene to gerbils or mice resulted in the vector infection of ependymal cells and the decrease of the food intake and body weight in the animals. Ependymal cells form a cell layer that separates the brain from ventricles, and in the third ventricle the cerebrospinal fluid and hypothalamic nuclei intimately interact. Since vectors of this invention can efficiently infect ependymal cells, they can be used to express a secretory protein in the ventricle so that the protein acts on hypothalamic nuclei (feeding center, satiety center, etc.). In addition, in an ischemic model using gerbils, it has been revealed that the cell injury is significantly reduced by introducing a viral vector for a growth factor expression into the hippocampus parenchymal cells, indicating a usefulness of the vector of this invention for preventing the cell death due to cell exfoliation in brain ischemia. These facts have indicated that vectors of this invention are useful as vectors for transfer of gene into the brain in various medical treatments.

The present invention relates to:

(1) A method for transferring nucleic acid into nerve cells, comprising a step of contacting the nerve cells with a negative-sense RNA viral vector or cells comprising said vector;

5 (2) A method of (1), wherein said nerve cells are the central nervous system cells;

(3) A method of (2), wherein said central nervous system cells are ventricular ependymal cells;

10 (4) A method of (2), wherein said central nervous system cells are hippocampus cells;

(5) A method of any one of (1) to (4), wherein nucleic acid contained in the negative-sense RNA viral vector comprises a foreign gene;

15 (6) A method of (5), further comprising allowing to transiently express said foreign gene;

(7) A method of (5), wherein said foreign gene encodes a secretory protein;

(8) A method of (7), wherein said protein acts on the hypothalamic nuclei;

(9) A method of (7), wherein said protein is capable of protecting the brain from ischemia;

20 (10) A method of (5), wherein said foreign gene is selected from the group consisting of FGF-1, FGF-5, NGF, CNTF, BDNF, GDNF, p35, CrmA, ILP, bc1-2 and ORF 150;

(11) A method for controlling the feeding behavior of animals, the method comprising administering a negative-sense RNA viral vector comprising FGF-1 or FGF-5 as a foreign gene to animals;

25 (12) A method for controlling the blood sugar level of animals, the method comprising administering a negative-sense RNA viral vector comprising FGF-1 or FGF-5 as a foreign gene to animals;

(13) A method of any one of (1) to (12), wherein said negative-sense RNA virus belongs to the Paramyxoviridae family;

30 (14) A method of (13) wherein said virus belonging to the Paramyxoviridae family is Sendai virus; and

(15) A negative-sense RNA viral vector used for transferring nucleic acid into nerve cells by the method of any one of (1) to (14).

In this invention, "negative-sense RNA viral vectors" include a complex that is derived from a negative-sense RNA virus and has the infectivity. Herein, "infectivity" means the "capability of a complex to transfer its nucleic acid or other substances inside thereof into a cell through its ability to adhere and fuse to the cell membrane".

35 In this invention, a negative-sense RNA viral vector can be prepared by using, for example, a negative-sense RNA virus as a starting material. Viruses used as starting materials are exemplified by, for example, viruses belonging to the Paramyxoviridae such as SeV, Newcastle disease virus, mumps virus, measles virus, RS virus (Respiratory syncytial virus), rinderpest virus and distemper virus; viruses belonging to the Orthomyxoviridae such as influenza virus;

viruses belonging to the Rhabdoviridae such as vesicular stomatitis virus and rabies virus; etc.

When SeV is used, a group of proteins encoded by three genes, NP, P/C and L, which are thought to be essential for its autonomous replication, are not necessarily required to be encoded by the viral vectors of this invention. For example, the vector of this invention can be produced in the host cells that carry the genes encoding this group of proteins so that these proteins are provided by the host cells. In addition, the amino acid sequences of these proteins are not necessarily identical to those native to the virus. Any mutations can be introduced, or substitutions by homologous genes from other viruses can be used as long as their nucleic acid-transferring activities are equal to or higher than those of the naturally occurring proteins.

Further, when SeV is used, a group of proteins encoded by the M, F and HN genes, which are thought to be essential for the disseminative capability of the virus, are not necessarily required to be encoded by the viral vectors of this invention. For example, the vector of this invention can be produced in the host cells that carry the genes encoding this group of proteins so that these proteins are provided by the host cells. In addition, the amino acid sequences of these proteins are not necessarily identical to those native to the virus. Any mutations can be introduced into the genes or substitution of the genes by homologous gene from other virus can be used as long as their nucleic acid transferring activities are equal to or higher than that of the naturally occurring proteins.

To transfer a foreign gene into nerve cells, a complex comprising a recombinant viral genome into which a foreign gene is inserted can be prepared and used. The complex comprising a recombinant viral genome can be obtained by means of *in vitro* or *in vivo* transcription of a modified cDNA derived from any of the aforementioned viruses or a recombinant virus thereof followed by reconstitution of the virus. A method for reconstituting a virus has already been developed (see WO97/16539).

In addition, instead of the complete SeV genome, incomplete viruses such as defective interfering particles (DI particles) (J. Virol. 68, 8413-8417, 1994), synthetic oligonucleotides, etc. may also be used as the component to constitute the complex.

When SeV is used as a material, a complex may contain all the three genes, M, F and HF, which are involved in the disseminative capability of the virus. However, in general, even though a complex comprising all the M, F and HN genes is transferred into the brain, the complex presumably fails to exhibit disseminative capability after formation of the viral particles, because of the absence of protease to cleave F protein, a protein essential for the disseminative capability of SeV. Herein, "disseminative capability" means "the ability of nucleic acid, which is transferred into a cell by infection or by employing an artificial technique, to replicate and direct the formation of infectious particles or their equivalent complexes which can disseminate the nucleic acid to other cells". However, to increase the safety, the genes involved in the disseminative capability of the virus are preferably eliminated or functionally inactivated in the

viral genome in the complex. In the case of SeV, genes involved in the disseminative capability of the virus are the M, F and/or HN genes. A reconstitution system of such complexes has been developed (WO97/16538). For example, for SeV, a viral vector comprising a genome from which the F and /or HN genes are deleted can be prepared from the 5 viral genome contained in the reconstituted complex. Such vectors are also included in the vectors of this invention for transferring nucleic acid into nerve cells.

The complex may contain on its envelope surface a factor that is capable of adhering to a specific cell, such as an adhesion factor, ligand, receptor, etc. For example, parts of the genes of a recombinant negative-sense RNA virus can be modified to inactivate the genes related to 10 immunogenicity or to enhance the efficiencies of transcription and replication of RNA.

RNA contained in the complex can incorporate a foreign gene at its appropriate site. To express a desired protein, a foreign gene encoding the protein is incorporated into the RNA. For the SeV RNA, a nucleotide sequence consisting of nucleotides in multiples of six is desirably inserted between the R1 and R2 sequences (Journal of Virology, 1993, Vol. 67, No. 8, pp. 4822-15 4830). Expression of the foreign gene inserted into the RNA can be regulated via the insertion site of the gene or the RNA sequence in the vicinity of the inserted gene. For example, in the case of SeV RNA, it is known that the nearer to the NP gene the insertion position of the RNA comes, the higher the expression level of the inserted gene becomes.

A foreign gene encoded by the RNA contained in the complex can be expressed by 20 infecting cells with the complex. As shown in the examples below, it has been demonstrated that a complex prepared as one embodiment of this invention by using the reconstitution system of SeV enables an efficient transfer of a foreign gene into various nerve cell strains. As shown in Example 5, it has also been revealed that another embodiment of the complex of this invention in which the  $\beta$ -glucuronidase gene is used as a foreign gene shows a significantly higher 25 expression level than retroviral vectors. Owing to these characteristics, the complex of this invention can be used for transferring genes into nerve cells. Since, one embodiment of the complex of this invention shown in Example 6 decreases its expression about one week after the intraventricular administration, it is useful in such a gene therapy that requires the gene expression of only for a limited period of time.

Nucleic acid or other compounds contained in the complex prepared can be introduced 30 into nerve cells by contacting the complex with nerve cells or by directly contacting the viral vector-producing cells with nerve cells. When the complex is administered into the brain, the administration can be performed, for example, by boring a hole on the cranial bone after craniotomy under anesthesia, followed by injecting the complex using a glass needle or the like 35 material. The complex can contain foreign genes. Foreign genes may include any types of genes, such as the nerve cell-specific gene, apoptosis-suppressing gene, other genes for treating various type of diseases, etc. Such genes can take the forms of antisense DNA and ribozyme so

as to inhibit the function of a specific gene.

For example, it has been revealed that the brain cell death in ischemic tissues does not occur soon after ischemia, but within several days after that (Neurosci. Lett. 1998, 240: 69-72). To prevent the brain cell death in such a case, a complex of this invention comprising a gene responsible for suppression of the cell death, such as bcl-2, etc. can be used. In fact, during the investigation whether administration of the vector of this invention could prevent the delayed exfoliation of fragile nerve cells due to depletion of nutrients caused by ischemia, it was revealed that administration of an FGF-1 expression vector could significantly prevent the cell exfoliation (Example 10). In addition, as demonstrated in Examples 6 and 8, the complex of this invention can transfer a foreign gene into ependymal cells and cells present along the ventricles via intraventricular administration. Use of a gene expressing a secretory protein as a foreign gene can diffuse the protein through the spinal fluid into the brain including the hippocampal area. As shown in Example 7, it is also possible to express a foreign gene in the pyramidal cells of the hippocampus by administering a complex of this invention into the cells. As shown in Examples 6 and 7, one embodiment of the complex of this invention was expressed in nerve cells of hippocampus even 13 days after the administration of the complex into the brain. The transfer of the complex did not cause serious cell exfoliation. These results indicate the usefulness of the complex of this invention for the gene therapy of central nerves. For example, in Example 9, it was demonstrated that the intraventricular administration of an FGF expression vector could successfully control the amount of food intake and reduce the body weight. Body weight loss attributable to FGF-2 (Denton, D. A. et al. (1995) Physiol. Behav. 57 (4): 747-752) and reduction of the blood sugar level accompanied with the body weight loss (Stephens, T. W. et al. (1995) Nature 377 (6549): 430-532) were already reported, which coincides with the results obtained in the present invention that the blood sugar level was reduced associated with the body weight loss.

Thus, the vectors of this invention provides a novel mode of vector administration targeting ependymal cells. In addition to ependymal cells, target cells include, but not limited to, cells present along the ventricles, cells in the hippocampal region, especially hippocampus pyramidal cells, neural stem cells, neural crest cells derived from mammalian embryos, etc. Genes that can be introduced include, but not limited to, those for fibroblast growth factors, nerve growth factors, apoptosis inhibitors, heat shock proteins, peroxidases, etc. Specific examples of such genes include those for FGF-1 (J. Biol. Chem. 271 (47): 30263-30271, 1996), FGF-5 (Proc. Natl. Acad. Sci. U.S.A. 87 (20): 8022-8026, 1990), NGF (Nature, 302 (2): 538-540, 1983), CNTF (Nature, 357 (6): 502-504, 1992), BDNF (EMBO J., 9 (8): 2459-2464, 1990; Genomics, 10 (3): 558-568, 1991), GDNF (J. Neurosci. Res. 41 (2): 279-290, 1995), p35 (J. Virol. 61 (7): 2264-2272, 1987), CrmA (Proc. Natl. Acad. Sci. U.S.A. 83: 7698-7702, 1986), ILP (EMBO J., 15 (11): 2685-2694, 1996), bcl-2 (Oncogene., 4 (11): 1331-6, 1989), ORP 150

(Biochem. Biophys. Res. Commun. 230 (1): 94-99, 1997), etc. Vectors of this invention are useful for not only searching genes by using DNA chips and DNA arrays, but also conveniently preparing model mice as well as developing medicines.

Animals into which the complex of this invention can be introduced include all kinds of

5 mammals such as human, gerbil, mouse, rat, rabbit, cattle, monkey, etc.

#### Brief Description of the Drawings

Figure 1 schematically shows a method for constructing the replication competent SeV comprising a foreign gene, such as for GFP or  $\beta$ -glucuronidase. Using primer 1, which has a 10 *NotI* site, and primer 2, which comprises, a transcription termination signal (R2), an intervening sequence (IG), a transcription initiation sequence (R1) and a *NotI* site, the ORF of a foreign gene is amplified by PCR and inserted into the *NotI* site of pUC18/T7HVJRz.DNA (+18).

Figure 2 is a frontal cross sectional view of the mouse brain showing the expression of GFP in a mouse infected with SeV vector comprising the GFP gene (GFP/SeV).

Figure 3 is a cross sectional view of the lateral ventricle showing the expression of  $\beta$ -glucuronidase in a  $\beta$ -glucuronidase-deficient mouse 3 days after the infection with SeV vector carrying the  $\beta$ -glucuronidase gene.

Figure 4A shows a cross sectional view of the lateral ventricle showing the  $\beta$ -glucuronidase expression (framed areas) in the ventricle of a  $\beta$ -glucuronidase-deficient mouse 20 12 days after the infection with SeV vector carrying the  $\beta$ -glucuronidase gene. Figure 4B shows the section adjacent to that of Figure 4A stained by Lorbacher method.

Figure 5 is a graph showing changes in the body weight of gerbils after the intraventricular administration of SeV expressing FGF-1, FGF-5 and GFP.

Figure 6 is a graph showing changes in the body weight of mice after the intraventricular 25 administration of Sendai virus expressing FGF-1, FGF-5 and GFP.

Figure 7 is a graph showing changes in the amount of food intake of mice after the intraventricular administration of SeV vector expressing FGF-1, FGF-5 and GFP.

Figure 8 is micrographs showing the delayed exfoliation of pyramidal cells in the hippocampal CA1 area of a gerbil 5 days after ischemia.

Figure 9 is micrographs showing the prevention of delayed exfoliation of pyramidal cells 30 in hippocampal CA1 region after the administration of a FGF-1 expressing Sendai viral vector.

#### Best Mode for Carrying out the Invention

The present invention will be explained in detail with reference to examples below, but is

35 not to be construed as being limited thereto.

Example 1. Preparation of the replication competent SeV

A *NotI* fragment comprising a foreign gene to be transferred, transcription initiation (R1) and termination (R2) signals, and intervening sequence (IG) (Fig. 1) was amplified by PCR and inserted into the *NotI* cleavage site of SeV transcription unit pUC18/T7HVJRz.DNA (+18) (Genes Cells, 1996, 1: 569-579) (Fig. 1). According to an established method (Genes Cells, 1996, 1: 569-579), using LLCMK2 cells and embryonated chicken eggs, the virus comprising the above-described genes was reconstituted, resulting in the recovery of the virus comprising the desired gene.

Example 2. Confirmation of infectivity of "GFP/SeV" to established nerve cell lines

As the established cell lines, rat phenochromocytoma (PC12), human neuroblastoma (IMR-32) and human glioblastoma cells (A172) were used. PC12 cells were cultured in a DMEM medium supplemented with horse serum and calf serum to a final concentration of 5% for each serum. To promote neurite outgrowth, a nerve growth factor (NGF7S) was added to the medium to a final concentration of 50 ng/ml. An MEM medium containing 10% calf serum supplemented with an MEM sodium pyruvate solution and MEM non-essential amino acid solution to the final concentrations of 1 mM and 0.1 mM, respectively, was used for the culture of human neuroblastoma cells (IMR-32). Human glioblastoma cells (A172) were cultured in a MEM medium (a high glucose medium) containing 10% calf serum.

$10^5$  cells were plated into a 6-cm dish containing NGF in the medium, incubated for 3 days to induce the neurite outgrowth and then used for PC12 cell infection experiment. After removing the medium, the cells were washed once with PBS. SeV into which a GFP gene is introduced (hereinafter referred to as GFP/SeV vector) was diluted with 500  $\mu$ l of PBS supplemented with 1% bovine serum albumin to  $10^6$  plaque forming unit (p.f.u.) , and was added to the cells to infect GFP/SeV vector for 20 min under the conditions where the cells were protected from drying. After the infection, the medium (5 ml) was added to the plates, and the cells were cultured for 2 days. After culturing, the cells were examined for GFP fluorescence under a fluorescence stereoscopic microscope. As a result, the infection of PC12 cells with GFP/SeV vector was confirmed by the GFP fluorescence within the cells. Fluorescence emission could not be observed with the control cells infected with SeV carrying no GFP gene and non-infected cells.

IMR-32 cells ( $3 \times 10^5$  cells) were plated into a 10-cm plate containing a predetermined medium, and cultured overnight. Based on the cell number estimated to be  $6 \times 10^5$  after the culture, GFP/SeV vector was diluted to m.o.i. (multiplicity of infection) of 10 with 1000  $\mu$ l of PBS containing 1% bovine serum albumin. After the cells were infected with the virus for 20 min, they were cultured in a predetermined medium for 12 or 36 h, and then examined for the GFP fluorescence under a fluorescence stereoscopic microscope. After the culture for 12 h, fluorescence was observed in the cell body of GFP/SeV-infected cells. After the 36-h culture,

GFP fluorescence was observed in the neurite portion in addition to the cell body. Fluorescence was not observed in the control cells infected with SeV carrying no GFP gene as well as non-infected cells.

A172 cells were also infected with the virus in a similar manner as that used for IMR-32

5 cells. Fluorescence was observed in the cell body of GFP/SeV-infected cells, but not in the control cells infected with SeV carrying no GFP gene as well as non-infected cells.

GFP/SeV vector infected all the established nerve cell strains used in the present study, and succeeded in expressing GFP from the GFP gene within the cells. These results indicated a possibility of the SeV infection of the primary culture of brain cells and of brain cells by *in vivo* 10 administration of the virus.

### Example 3. Culture of the primary rat brain cells

An SD rat of 18-day pregnancy was deeply anesthetized with diethyl ether, and euthanized by the exsanguination from the axillary artery. After the abdominal region was disinfected with 15 95% ethanol, it was subjected to laparotomy to remove the fetuses together with the womb.

Subsequent procedures were all performed under the germfree conditions on ice, or in ice-cold 20 solutions unless otherwise stated. Fetuses was removed from the womb using scissors and roundheaded forceps, and transferred to a plate containing 20 ml of an operation solution (50% DMEM and 50% PBS). After the fetuses were placed on a sterilized gauze pad, their scalp and skull were incised along the midline using two pairs of INOX#4 forceps. Subsequently, a pair 25 of INOX#7 forceps was inserted along the undersurface of the brain tissue to scoop up the brain tissue as a whole with the medulla oblongata being cut off, and the tissue was excised and placed in the operation solution. Under a stereoscopic microscope, the brain in the operation solution was filleted into three portions using two scalpels to separate the brain stem, and two pieces of cerebral hemispheres containing the hippocampus and corpus striatum were transferred into another operation solution with roundheaded forceps. Under a stereoscopic microscope, the meninx was completely removed from the surface of the brain tissue using two pairs of INOX#5 forceps, and transferred into another operation solution using roundheaded forceps for washing. Six pieces of cerebral hemispheres were placed into a preservation solution (90% DMEM

30 (containing 5% horse serum and 5% calf serum), and 10% DMSO) with roundheaded forceps, and then they were cut into small pieces less than 1 mm using a scalpel on slides. The tissue pieces thus cut were placed into about 1.5 ml of the preservation solution in a pre-cooled tube, which was stored in a freezing container, frozen slowly over a period of 3 hours, and then stored in liquid nitrogen. The tissue pieces of 6 cerebral hemispheres were taken out from the liquid 35 nitrogen, thawed at 32°C, washed twice in 8 ml of the operation solution, and allowed to stand for 30 sec, and then the supernatant was removed. To the tissue pieces were added 5 ml of an ice-cold papain solution (papain 1.5 U, cysteine 0.2 mg, bovine serum albumin 0.2 mg, glucose 5

mg, and DNase 0.1 mg/ml) which had been filtered and sterilized. The mixture was warmed at 32°C for 15 min and mixed by inverting the tube every 5 min. The supernatant was separated, and 5 ml of a solution containing 20% calf serum were added. A papain solution (5 ml) preheated to 32°C was added to the precipitate fraction, and the resulting mixture was further  
5 warmed for 15 min. The mixture was mixed by inverting the tube every 5 minutes. After good turbidity of the supernatant as well as translucence of the tissue pieces were confirmed, the tissue pieces were split by pipetting. The first supernatant fraction preheated to 32°C was added to this sample solution, and the resulting mixture was centrifuged in a centrifuge preheated to 32°C (at 1200 rpm for 5 min). After removal of the supernatant, 5 ml of DMEM (containing  
10 5% horse serum and 5% calf serum) were added to and mixed with the residue to break the cells up, followed by centrifugation under the above-described conditions. After the removal of supernatant, 2 ml of DMEM (containing 5% horse serum and 5% calf serum) were added to the residue, and the resulting mixture was stirred. As a result of cell counting, the cell number was found to be  $5 \times 10^6$  cells/ml. The primary culture of brain cells thus obtained were seeded on a  
15 polyethylene imine-coated plate and cultured.

Example 4. Confirmation of infectivity of SeV to the primary culture of brain cells using GFP/SeV vector

The primary culture of brain cells obtained in Example 3 was cultured in a 10-cm plate for  
20 3 days. After the removal of the supernatant, a sample solution prepared by diluting GFP/SeV vector in 1000  $\mu$ l of PBS containing 1% bovine serum albumin was added to the culture to infect with the virus for 20 min. After the infection, 10 ml of DMEM medium (containing 5% horse serum and 5% calf serum) was added, and the cells were cultured for 2 days. The cells were then examined for the fluorescence of GFP under a fluorescence stereoscopic microscope.  
25 Almost all the cells displayed fluorescence. That is, it was confirmed that SeV infects even the primary culture of brain cells.

Example 5. Infection of SeV vector carrying the  $\beta$ -glucuronidase gene (hereinafter abbreviated as  $\beta$ -glu/SeV) to human fibroblast cells deficient of the  $\beta$ -glucuronidase gene  
30 and expression of said enzyme in the cells

For the implementation of this invention, human fibroblast cells deficient of the  $\beta$ -glucuronidase gene (hereinafter abbreviated as  $\beta$ -glu-deficient cell) and human normal fibroblast cells were used.

Mucopolysaccharidosis type VII, one type of mucopolysaccharidosis, is caused by  
35 deficiency of  $\beta$ -glucuronidase, and shows a variety of clinical symptoms ranging from a mild case to severe case with fetal hydrops. There are many severe cases showing various symptoms developed during the infantile period, including characteristic facial feature, splenohepatomegaly,

psychomotor retardation, bone deformation, etc.

It has been indicated that, for the intracellular transport of  $\beta$ -glucuronidase to lysosome, the addition of sugar chain to the enzyme molecule and the phosphorylation of the 6-position of the mannose moiety of the enzyme are necessary. On the arrival at lysosome, C-terminus of the  
5 enzyme undergoes proteolysis.

Prior to the implementation of this invention,  $\beta$ -glu/SeV vector was examined for 1) its infectivity to human fibroblast cells, 2) its expression amount, and 3) the presence of its molecular species to be transported to lysosome.

1)  $\beta$ -glu deficient fibroblast cells were prepared so that  $10^5$  cells/well were placed in a  
10 6-well plate.  $\beta$ -glu/SeV vector was diluted in 100  $\mu$ l of PBS containing 1% bovine serum albumin so that the multiplicity of infection (m.o.i.) became 5, and the overnight-cultured  $\beta$ -glu deficient cells were infected for 1 h. The cells were cultured in a serum-free MEM medium for  
15 24 h. The cells thus cultured were fixed in a mixture of formalin and acetone (1:7, v/v). With naphthol AS-BI glucuronide as a substrate, the reaction was performed in the acetate buffer, pH 5.0, at 37°C, and the substrate decomposition was monitored by the red coloration. As a result, the cytoplasm of  $\beta$ -glu deficient cells incubated with " $\beta$ -glu/SeV" was stained red, indicating  
15 that  $\beta$ -glu deficient cells were infected with " $\beta$ -glu/SeV" to express the transferred gene.

2)  $\beta$ -glu deficient cells were prepared so that  $10^5$  cells/well were placed in a 6-well  
20 plate. " $\beta$ -glu/SeV" was diluted in 100  $\mu$ l of PBS containing 1% bovine serum albumin so that the multiplicity of infection (m.o.i.) became 0.1 and 1.0, and incubated with overnight-cultured  $\beta$ -glu deficient cells for 1 h. The cells were cultured in a serum-free MEM medium for 24 or 48 h. After the incubation for the predetermined period of times, cells were recovered  
25 and sonicated to prepare intracellular fractions. With 4-methylumbelliferyl- $\beta$ -D-glucuronide as a substrate, the amount of 4-methylumbelliferone (MU), the enzymatic reaction product, was determined by measuring the fluorescence intensity with a fluorospectrophotometer. The results are shown in Table 1. In this table, the expression amount was represented by the amount of 4-methylumbelliferone (MU) produced by 1 mg of protein in the intracellular fraction in 1 h.

Table 1

| Cell                              | Infecting condition                      | Amount of expression<br>(nmol MU/mg total protein/h) |
|-----------------------------------|--|--|
| $\beta$ -glu-deficient fibroblast | No infection                             | 53   |
| Normal fibroblast                 | No infection                             | 276  |
| $\beta$ -glu-deficient fibroblast | $\beta$ -glu/retro                       | 911  |
| $\beta$ -glu-deficient fibroblast | $\beta$ -glu/SeV<br>(m.o.i. = 0.1, 24 h) | 15,900   |
| $\beta$ -glu-deficient fibroblast | $\beta$ -glu/SeV<br>(m.o.i. = 1.0, 24 h) | 27,100   |
| $\beta$ -glu-deficient fibroblast | $\beta$ -glu/SeV<br>(m.o.i. = 0.1, 24 h) | 21,100   |
| $\beta$ -glu-deficient fibroblast | $\beta$ -glu/SeV<br>(m.o.i. = 1.0, 24 h) | 32,300   |

As shown in Table 1, the expression amount ranged 15,900-32,300 (nmol MU/mg total protein/h), and 276 for normal fibroblast cells and 911 for the cell expressing  $\beta$ -glucuronidase with a retrovirus ( $\beta$ -glu/retro), indicating that SeV strongly expresses a transgene in the SeV-infected cells.

3) The fractions obtained in 2) were used as the intracellular fraction of " $\beta$ -glu/SeV"-infected- $\beta$ -glucuronidase-deficient-fibroblast cells. As the culture supernatant fraction, proteins contained in the culture supernatant were recovered by precipitation with cold acetone. Test samples thus obtained were subjected to Western blot analysis using an anti-human  $\beta$ -glucuronidase antibody. As a result, in the intracellular fraction of " $\beta$ -glu/SeV"-infected- $\beta$ -glucuronidase-deficient-fibroblast cells, two types of proteins were identified; one has high molecular weight and another has low molecular weight, and both are reactive with the anti-human  $\beta$ -glucuronidase antibody. The band of the low molecular weight protein corresponds to that of the protein reactive with the anti-human  $\beta$ -glucuronidase antibody in normal fibroblast cells, indicating that it is a molecular species of  $\beta$ -glucuronidase the C-terminus of which has undergone proteolysis after transported to lysosome. The high molecular weight protein was not observed in the normal fibroblast cell, but present in the intracellular and supernatant fractions of  $\beta$ -glu/SeV-infected- $\beta$ -glucuronidase-deficient-fibroblast cells. The supernatant fraction contained only the high molecular weight protein. This may be due to too high an expression of  $\beta$ -glucuronidase caused by  $\beta$ -glu/SeV vector infection, in which transport of the high molecular weight protein species to lysosome failed to catch up with such a high enzyme expression, resulting in the secretion of the protein into microsomes or extracellular

space. Alternatively, judging from its molecular weight, the high molecular weight protein may be a molecular species with a sugar chain attached but without the 6-position of mannose moiety being phosphorylated so that it cannot be transported to lysosome.

Thus, the human  $\beta$ -glucuronidase, which is assumed to be transported to lysosome, was able to be expressed in the intracellular fraction of  $\beta$ -glu/SeV-infected- $\beta$ -glucuronidase-deficient-fibroblast cells.

**Example 6. Expression of GFP in ependymal cells by intraventricular administration of GFP/SeV**

Mice of 8-10 weeks old were anesthetized with 200  $\mu$ l of 10-fold diluted Nembutal. After craniotomy, a hole of 1 mm in diameter was bored in the skull at the position 1.0 mm from the bregma and 1.5 mm to the right of the medline with a dental drill. After the removal of the dura, GFP/SeV vector was administered at the position 1.3 mm deep using a 27 G syringe needle. The dose of GFP/SeV vector was 20 to 30  $\mu$ l, and the number of the virus contained in the sample solution was estimated  $1 \times 10^7$  p.f.u. to  $1.5 \times 10^7$  p.f.u. Control mice were administered PBS or SeV carrying no GFP gene. Autopsy was performed 3, 5, 7 and 10 days after the administration. A whole brain was removed, and a frontal cross section was made. Under a stereoscopic fluorescence microscope, GFP fluorescence was observed. In the dissected brain autopsied 3 days after the administration of GFP/SeV vector, the conspicuous GFP fluorescence was observed. At the site along the ventricle of the frontal cross section, distinct fluorescence of GFP was observed (Fig. 2). As described in Example 8 below, SeV-infected cells emitting GFP fluorescence were thought to be ependymal cells. The cells along the lateral ventricle also became fluorescent 5 and 7 days after the infection. However, the fluorescence intensity was significantly decreased in the cells 7 days after the infection, and no fluorescent brain cells could be observed 10 days later. Fluorescence could not be observed in the control mouse brains to which PBS or SeV carrying no GFP gene had been administered as a control.

**Example 7. Administration of GFP/SeV vector to brain parenchyma under stereotaxy**

To examine the SeV infection of nerve cells, especially pyramidal cells of hippocampus, which is the main object of this invention, precisely targeted administration of SeV to the vicinity of hippocampus is required. Therefore, a stereotaxy was conducted to introduce SeV into the brain parenchymal and the brain parenchyma cells were examined for the infection. As the experimental animals, 1) mouse and 2) rat were used.

1) Two holes of 1 mm in diameter each were bored through the skull at the position 2 mm to the left and right of the medline and 3 mm anterior to the bregma using a dental drill. GFP/SeV vector ( $1.5 \mu$ l each) was administered to the parenchymal portions, 3.5 mm deep on the right side and 2.5 mm deep on the left side, using a glass capillary. The skull was closed,

and surgically opened 3 days later to examine the GFP expression, which was observed in the parenchymal portion. After the fixation with ethanol, frozen tissue slices were prepared. Although GFP fluorescence was significantly reduced in the frozen slices after ethanol fixation due to the outflow of chromophores, fluorescent sites were still observed. In the white matter near the internal capsule, GFP fluorescence was observed on the axon from which myelin protein was eluted with ethanol. Furthermore, GFP fluorescence was also observed in the axon in the area presumed to be the corpus striatum.

These results demonstrated that GFP/SeV vector was capable of infecting nerve cells of the mouse brain.

2) Since a precise stereograph has already been made for rat, GFP/SeV vector can be accurately administered to the vicinity of pyramidal cells in the hippocampus CA1 area. A rat weighing about 170 g was anesthetized, and, after craniotomy, two holes of 1 mm in diameter each were bored through the skull at the positions 2 mm to the left and right of the medline and 4.5 mm anterior to the internal (sigma) with a dental drill. GFP/SeV vector (1.5  $\mu$ l each) was administered to the parenchymal portions, 3.5 mm deep on the right side and 2.5 mm deep on the left side, using a glass capillary. The skull was closed, and surgically opened 3 days later to examine the GFP expression. As a result, the GFP expression was observed in the hippocampus CA1 pyramidal cell area, where GFP/SeV vector was administered in 2.5 mm deep. Enlarged view of the region adjacent to the hippocampus by fluorescence microscopy revealed the marked fluorescence in the cell bodies of the hippocampus CA1 pyramidal cells and dendrites. The GFP expression was observed even in the pyramidal cells 13 days after the administration. Even 13 days after the administration of GFP/SeV, the GFP expression was observed in the cell bodies and dendrites of the pyramidal cells. These results demonstrate that SeV infection does not cause the nerve cell death even 13 days after the infection, strongly suggesting the usefulness of Sev as a vector for the gene therapy directed to prevention of the exfoliated cell death following the brain ischemia.

Example 8. Gene therapy trial on  $\beta$ -glucuronidase-deficient mice using  $\beta$ -glu/SeV vector

The results of Example 6 indicates that the ependymal cells are infected with SeV by intraventricular administration. Therefore, the inventors conducted an experiment in which  $\beta$ -glu/SeV vector is administered to  $\beta$ -glucuronidase-deficient mice (J. Clin. Invest., 1989, 83: 1258-1266) to induce secretion of  $\beta$ -glucuronidase from the infected cells into the cerebrospinal fluid and then to be taken up by target cells so that the symptoms would be improved.

Homozygous mice were selected from mice obtained by breeding heterozygous mice based on the  $\beta$ -glucuronidase activity in the tail vein blood of the mice and on the presence of the *Nla*IV cleavage site in the PCR amplification fragments of the  $\beta$ -glucuronidase gene-

deficient site on the chromosomes of the mice, and were used in the present experiment. Administration of  $\beta$ -glu/SeV vector was carried out according to the method described in Example 6. The brain was excised 3 or 12 days after the administration to prepare the frozen tissue slices. The  $\beta$ -glucuronidase activity in the tissue was assayed using a modification of the method described in Example 5, 1). As shown in Figure 3, the sites at which  $\beta$ -glucuronidase was expressed were strongly stained red along the ventricles. When magnified by microscopy, the ependymal cells of the lateral ventricle were verified to strongly express  $\beta$ -glucuronidase, which was then secreted from the cells. On the tissue slice prepared 12 days after the administration (Figure 4),  $\beta$ -glucuronidase that had been expressed in and then secreted from the ependymal cells of the lateral ventricle was shown to be diffused into the ventricle with the migration of the spinal fluid to reach the vicinity of the hippocampus. Physical capabilities of the homozygous mice was apparently improved, although slightly, by this administration.

15 Example 9. Experiments on eating depression caused by administration of the Sendai viral vector carrying FGF-1 or -5 (eating depression experiments in gerbils and mice)

Gerbils (weighing 60 to 80 g) were anesthetized with Nembutal, fixed to a stereotactic instrument, depilated, and then incised in the scalp along the medline. A hole was bored in the skull at the position 1.0 mm from the bregma and 1.5 mm to the right of the medline using a dental drill with care to avoid damaging the blood vessels under the cranial bone. After drilling the hole, the dura and others were removed with tweezers. Mouse FGF-1/SeV vector ( $5 \times 10^6$  pfu), human FGF-5/SeV vector ( $1 \times 10^7$  pfu) and GFP/SeV vector ( $5 \times 10^6$  pfu) were injected 1.0 mm deep into the right lateral ventricle ( $n = 2$ ) with a 30G syringe needle. The recombinant viruses were prepared according to Example 1. Changes in the body weight were monitored by measuring the weight, and decrease in the body weight was observed from the next day of the administration (Figure 5). In the FGF-1-administered group, the body weight started to decrease from the next day of the administration, and continued to decrease by about 5% everyday till 5 days later, resulting in a 29.5% decrease 6 days later, and the maximum decrease of 29.8% was observed 7 days later. Then, the body weight turned to increase, and was recovered to a 3.5% decrease 20 days after the administration. In the FGF-5 administered group, the body weight started to decrease from the next day, reached the maximum of 21.7% decrease 5 days after the administration, and then turned to increase, being recovered to a 8.0% decrease 20 days later. In the FGF-9 administered group, similar decrease in the body weight was observed from the next day, showed the maximum of 22.9% 5 days after the administration, and then turned to increase, being recovered to a 6.40% decrease 20 days later. In the control group to which GFP/SeV was administered, the maximum of a 5.8% decrease in the body weight, which was presumably caused by the administration itself, was observed. However, the rate of

the body weight loss was relatively small as compared with the FGF-administered groups, clearly indicating that FGF affects the body weight loss.

Since the body weight decrease due to the administration of FGF-1/SeV vector and FGF-5/SeV vector was observed in gerbils, more-detailed study was performed using B-6 mice 5 (weighing about 20-22 g). The right lateral ventricle was selected as the administration site, and a hole of 1.0 mm in diameter was bored in the skull at the position 1.0 mm from the bregma and 1.5 mm to the right of the medline with a dental drill. After the removal of the dura, a sample was administered to the animal in the hole at the depth of 1.3 mm with a 27G-syringe 10 needle. The sample solutions were prepared by adding 9  $\mu$ l, 8  $\mu$ l and 9  $\mu$ l of PBS to 1  $\mu$ l of FGF-1/SeV vector ( $1 \times 10^6$  pfu), 2  $\mu$ l of FGF-5/SeV vector ( $2 \times 10^6$  pfu), and 1  $\mu$ l of control GFP/SeV vector ( $1 \times 10^6$  pfu) solutions, respectively. The body weight and the food intake were monitored for 2 weeks after the viral administration.

The control mice administered with GFP/SeV showed no decrease in the body weight, but showed a 7.5% increase as compared with the weight measured prior to the administration 15 (Figure 6). The amount of the food intake was also not significantly changed (Figure 7). In the FGF/SeV-administered group, an average 30.5% decrease in the body weight was observed 6 days after the administration (Figure 6). Then, the body weight turned to increase, resulting in a 13.5% decrease weight 2 weeks later. The change in the amount of food intake due to the FGF-1 administration was so dramatic that almost no food intake was observed from day 2 to day 6, especially from day 3 to day 6 after the administration (Figure 7). In the FGF-5/SeV vector administered group, although the decrease in the body weight was also observed, the rate of decrease was smaller as compared with the FGF-1/SeV-administrated group, and a 17.9% decrease at the maximum (Figure 6). The effect on the body weight decrease was in a tendency similar to that obtained in the gerbil experimental system. Although the effect of the FGF- 20 5/SeV vector administration on the body weight decrease was smaller than that of the FGF-1/SeV vector administration, the decrease in the food intake was clearly observed (Figure 7).

As shown in the results of the example, the effect of the intraventricular expression of FGF induced by SeV vector on the body weight decrease was a 30% decrease at the maximum. Considering that the effect of the intraventricular injection of FGF in the purified protein form on 30 the decrease in the body weigh was 7 to 8% at most, the rate of 30% achieved in the present invention was shown to be extremely high. Difference in these effects may be due to the difference in the intraventricular accumulation of FGF depending on the administration methods, but there is another possibility that this difference is due to a direct action of FGF on nerve cells through the SeV vector infection to ependymal cells. As to the feeding control in the brain, only 35 the control by the nerve nuclei of hypothalamus has been reported. In view of this, it is inferred that SeV vector efficiently infects ependymal cells to secrete a functional protein into the cerebrospinal fluid in the ventricle, and that said secretory protein efficiently acts on the

hypothalamic nerve nuclei to exert the feeding control. This inference would be supported by the facts that a part of the hypothalamic nerve tissue has a nerve construction with the tight junctions of the blood-brain barrier being lost and contains neurons to receive liquid factors in the peripheral circulation and cerebrospinal fluid.

5 Among the hypothalamic nuclei, chemosensitive neurons are present in the ventromedial hypothalamus (VMH) and lateral hypothalamic area (LHA), which are thought to be the feeding and satiety centers, and the neuron activity alters in response to metabolic products and hormones contained in blood and cerebrospinal fluid. These VMH and LHA neurons respond to glucose, and certain cytokines and growth factors are also known to function as  
10 appetite regulators. In addition, it has been demonstrated that, from the disruption experiment, the paraventricular nucleus (PVN) is also responsible for suppression of food intake. This nucleus has neurons that produce corticotropin releasing hormone (CRH) and shows the eating depression and activation of sympathetic nerve activity. Furthermore, the arcuate nucleus (ARC) is the site to produce NPY, a food intake stimulator, which is suggested to target PVN.  
15 The results of the experiments on the control of eating behavior described herein suggest that FGF acted on the nerve nuclei. Attention should be paid on the relation with leptin, which is expressed in mature adipocytes having lipid droplets and has been extensively studied in relation to eating behaviors as well as NPY, etc.

20 Example 10. Experiment on suppression of ischemic cell exfoliation by using gerbils

The area exposed to brain ischemia undergoes cell damage, and is further led to the cell death as the ischemia progresses. The extent of cell death depends on the degree and duration of ischemia. In the case of severe ischemia, not only nerve cells but also all constitutive cells in the ischemic area sustain irreversible injuries in a short period of time, resulting in the formation 25 of brain infarction focus caused by necrosis. However, in the case of severe ischemic stress of short duration, or in the case of slight ischemia of long duration, the cells in the ischemic region become fragile depending on the severity of ischemia. The most fragile cells are nerve cells, and then oligodendrocytes follow. Astroglia, microglia, and vascular endothelial cell have been known to be more resistant to the ischemic stress. From the examination using a diffuse brain 30 ischemia model, it has been known that there are differences in the resistance to ischemic stress among nerve cells. The known most fragile cells include nerve cells of the hippocampus CA1, those of the hilum of dentate gyrus, and those of the vestibular nuclei in the occipital region of head, which show a delayed cell death. The delayed nerve cell death is a good model of selective nerve cell death with high reproducibility independent of the energy insufficiency, 35 contributing a great deal to the elucidation of molecular mechanisms of ischemic cell death. There have been many reports on the experiments using these model systems to examine, for example, what cascade the nerve cells may go through to their death, which step of the cascade is

critical to protect the cell, into what type of cell death the delayed nerve cell death is classified, etc.

As the experimental model animals, rats, gerbils and mice are often used. These animals are used to study and treat the pathologic changes in the portions vulnerable to ischemia, such as hippocampus, corpus striatum, etc., induced by causing transient ischemia in the whole brain of the ischemia models for several to several ten minutes. A rat four vessel occlusion model, a rat hypotensive bilateral common carotid artery occlusion model, a bilateral common carotid artery occlusion model of gerbils, etc. are frequently used as the ischemia model. The present inventors carried out an ischemia experiment using a bilateral common carotid artery occlusion model of gerbil. It has been known that in gerbils cell death occurs mainly in most of the pyramidal cells in the hippocampal CA1 area when animals are subjected to a short time (5 min) ischemia. Therefore, the present inventors performed an experiment aiming at prevention of the cell exfoliation after ischemia by introducing into SeV a gene capable of preventing the cell death and administering the resulting complex to the hippocampus of gerbils.

15 <Preparation of an ischemic cell death model of gerbil>

Experiments were carried out with a bilateral common carotid artery occlusion (5 min) model of gerbil. By occluding (for 5 min) the bilateral common carotid artery of a gerbil, the pyramidal cells of hippocampus are selectively exfoliated 3-5 days after the occlusion. However, since this phenomenon is not commonly observed among gerbils, it is necessary to screen gerbils excellent as a model animal from those obtained from a commercial source. The gerbils selected by the screening (obtained from Instructor Dr. Maeda, Department 1 of Anatomy, Osaka City University) were used for the experiment.

20 After anesthetized with ketamine, the animals were subjected to thoracotomy to find out the carotid arteries on the left and right sides of the trachea, and fat adhering to the carotid artery was removed. After the fat removal, the carotid arteries were occluded for 5 min with clips. During this procedure, since the rate of nerve cell death is significantly reduced when the brain and body temperatures are low, the animals were kept warm to retain the body temperature at 37.5°C being monitored with a thermometer inserted into the anus. The clips were removed 5 min later, and the blood was perfused again. Five days later, the gerbils were sacrificed, and, after the craniotomy, the brain was excised to prepare tissue slices in paraffin. Conditions of nerve cells were confirmed by toluidine staining. As expected, the exfoliation of the pyramidal cells was observed in the hippocampal CA-1 area (Figure 8). Thus, the ischemic cell death model of gerbil has been prepared.

35 <Experiment on prevention of nerve cell death by introduction of the recombinant SeV>

The SeV vector prepared above is used to examine whether the Sev vector is effective for

preventing the nerve cell as follows: On the day before ischemia, the virus is introduced into only the right brain of the gerbils. Ischemia is applied on the next day, and the animals are sacrificed 5-6 days later to observe the hippocampus pyramidal cells.

5 <Transfer of FGF-1/SeV into hippocampus>

Gerbils weighing 60-80 g were selected and used in this experiment. After anesthetized with Nembutal, the animals were fixed to a stereotactic instrument. The brain was then depilated and the scalp was cut open along the midline of the brain. A hole was bored through the skull at the position 5 mm from the bregma and 2 mm to the right of the midline using a dental drill with care not to damage the blood vessels under the cranial bone. After drilling the hole, the dura and others were removed with tweezers. An administration glass needle was inserted into the position at the depth of 1.4 mm, and the animals were allowed to stand for 2 min. Through the glass needle, 0.5 to 1.0  $\mu$ l of an FGF-1/SeV vector solution (vector of  $1.0 \times 10^6$  pfu to  $2.0 \times 10^6$  pfu) was injected to the position in a period of 12 min, and the animal was allowed to stand for further 10 min. The needle was removed, and the incision was sewed up. In this procedure, the virus was administered only to the right brain, and the exfoliation of nerve cells after ischemia was determined by comparing the right and left brains.

<Ischemic operation>

After anesthetized with ketamine, the animals were subjected to thoracotomy to find out the carotid arteries on the left and right sides of the trachea, and fat adhering to the carotid arteries was removed. After the fat removal, the carotid arteries were occluded for 5 min with clips. During this procedure, since the nerve cell death is significantly reduced when the brain and body temperatures are low, the animals were kept warm to retain at the body temperature at  $37^\circ\text{C}$ , being monitored using a thermometer inserted into the anus. The clips were removed 5 min later, and the blood was perfused again. Five to six days later, the animals were sacrificed.

<Preparation of paraffin sections>

After the animal was sacrificed, frontal cross sections of the hindbrain were made into 300-500  $\mu\text{m}$  thick slices, soaked in 4% paraformaldehyde overnight, and embedded in paraffin with an automatic apparatus for fixation and embedding. The sections (5  $\mu\text{m}$  thick) were prepared, deparaffinized, and subjected to immunohistochemical staining and other stainings.

<Immunohistochemical staining>

Sections of the FGF-1-administered brain were prepared to examine for the reactivity to an antibody against the virus, to an anti-tubulin antibody (to determine the effect of ischemic operation), to an anti-GFAP antibody (to examine the astrocyte movement), and to an apoptag

antibody (to examine the presence of apoptosis). The results are briefly summarized as follows (Table 2).

Table 2

5

| Determinations of the effect of FGF-1                 | Antibody                | Determination |
|---|-------------------------|---------------|
| Introduction of the virus into the hippocampal area   | anti-virus antibody     | ○             |
| Determination of the effect of the ischemic operation | Anti-β tubulin antibody | ○             |
| Morphology of the soma                                | HE staining             | ○             |
| Movement of astrocytes                                | Anti-GFAP antibody      | ○             |
| Presence of apoptosis                                 | Apoptag                 | ○             |

In the pyramidal cells of the hippocampal CA-1 region, HE staining did not reveal any changes in the nerve cells in the control sample, which underwent no ischemia. Many of the cells in one side of the brain which underwent ischemia but were not administered with the virus were atrophic nerve cells displaying nuclear condensation in the nucleus and eosinophilic change in the cytoplasm, so-called ischemic changes. In contrast, in the other side of the brain, which underwent ischemia and was administered with the virus, a small number of deformed nerve cells were observed to be dispersed, but a majority of the nerve cells retained the original morphology. On the side to which the virus was administered, a region that was positive for the antibody against the virus was observed. In the nerve cells that underwent ischemia but were not administered with the virus, the most of the cells that showed deformation were positive for the apoptag-staining. In contrast, in the cells which underwent ischemia and were administered with the virus, only a very few cells that stained with HE and showed the morphological change were positive for the apoptag-staining, indicating that apoptosis was suppressed in the majority of the cells in this side (Figure 9).

#### Industrial Applicability

The present invention has provided a method for transferring a gene into nerve cells in the tissues including the central nervous tissue, into which transfer of a gene has hitherto been difficult. Use of the method of this invention enables the efficient transfer of a desired gene into the cells in gene therapy, etc.

## CLAIMS

1. A method for transferring nucleic acid into nerve cells, comprising a step of contacting the nerve cells with a negative-sense RNA viral vector or cells comprising said vector.

5 2. A method of claim 1, wherein said nerve cells are the central nervous system cells.

3. A method of claim 2, wherein said central nervous system cells are ventricular ependymal cells.

4. A method of claim 2, wherein said central nervous system cells are hippocampus cells.

10 5. A method of any one of claims 1 to 4, wherein nucleic acid contained in the negative-sense RNA viral vector comprises a foreign gene.

6. A method of claim 5, further comprising allowing to transiently express said foreign gene.

7. A method of claim 5, wherein said foreign gene encodes a secretory protein.

8. A method of claim 7, wherein said protein acts on the hypothalamic nuclei.

9. A method of claim 7, wherein said protein is capable of protecting the brain from ischemia.

10. A method of claim 5, wherein said foreign gene is selected from the group consisting of FGF-1, FGF-5, NGF, CNTF, BDNF, GDNF, p35, CrmA, ILP, bc1-2 and ORF 150.

11. A method for controlling the feeding behavior of animals, the method comprising administering a negative-sense RNA viral vector comprising FGF-1 or FGF-5 as a foreign gene to animals.

12. A method for controlling the blood sugar level of animals, the method comprising administering a negative-sense RNA viral vector comprising FGF-1 or FGF-5 as a foreign gene to animals.

25 13. A method of any one of claims 1 to 12, wherein said negative-sense RNA virus belongs to the Paramyxoviridae family.

14. A method of claim 13 wherein said virus belonging to the Paramyxoviridae family is Sendai virus.

30 15. A negative-sense RNA viral vector used for transferring nucleic acid into nerve cells by the method of any one of claims 1 to 14.

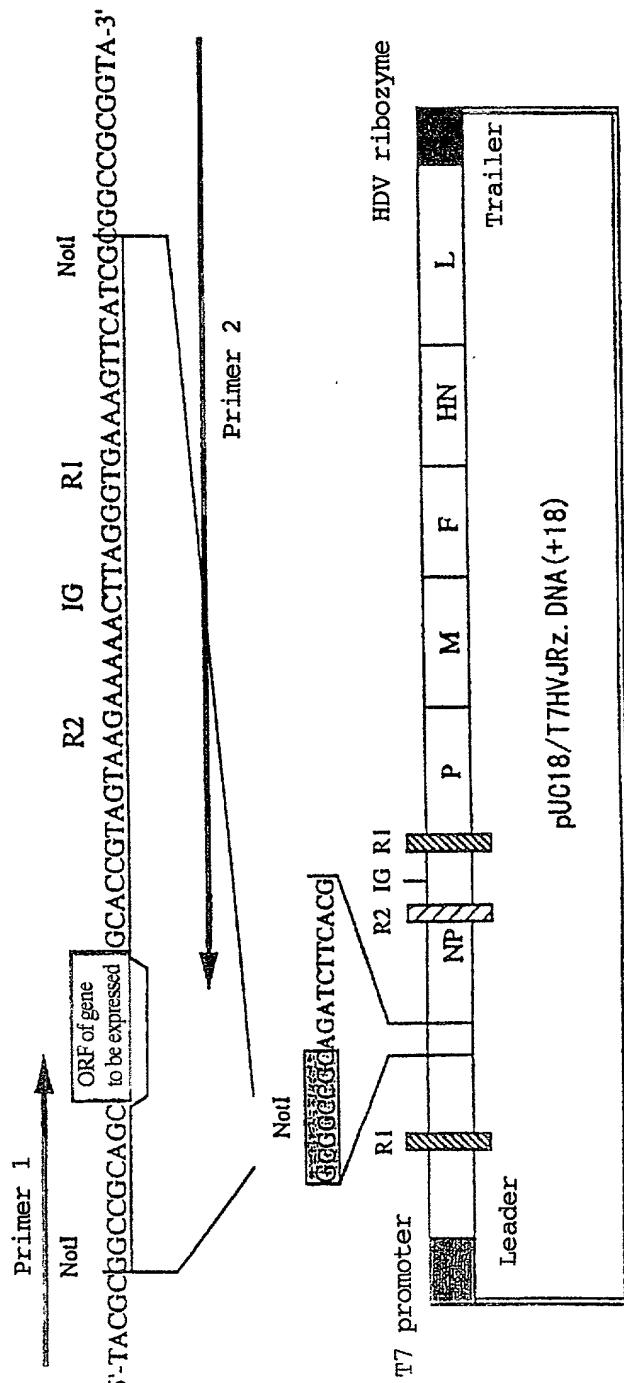
## ABSTRACT

Use of a negative-sense RNA virus vector has enabled transfer of nucleic acid into nerve cells. The method of this invention can be used for introducing a gene efficiently into nerve cells including the central nerve tissue in gene therapy, etc.

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Figure 1



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Figure 2



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Figure 3



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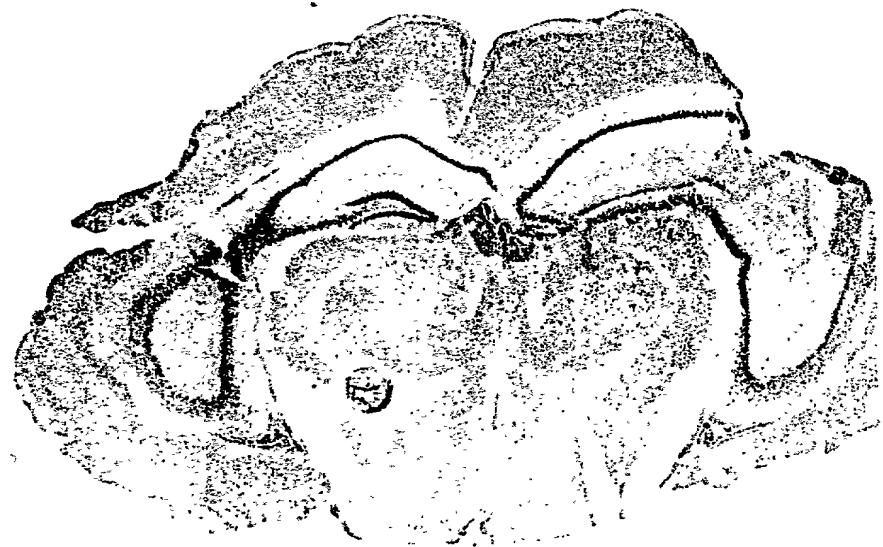
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Figure 4

A



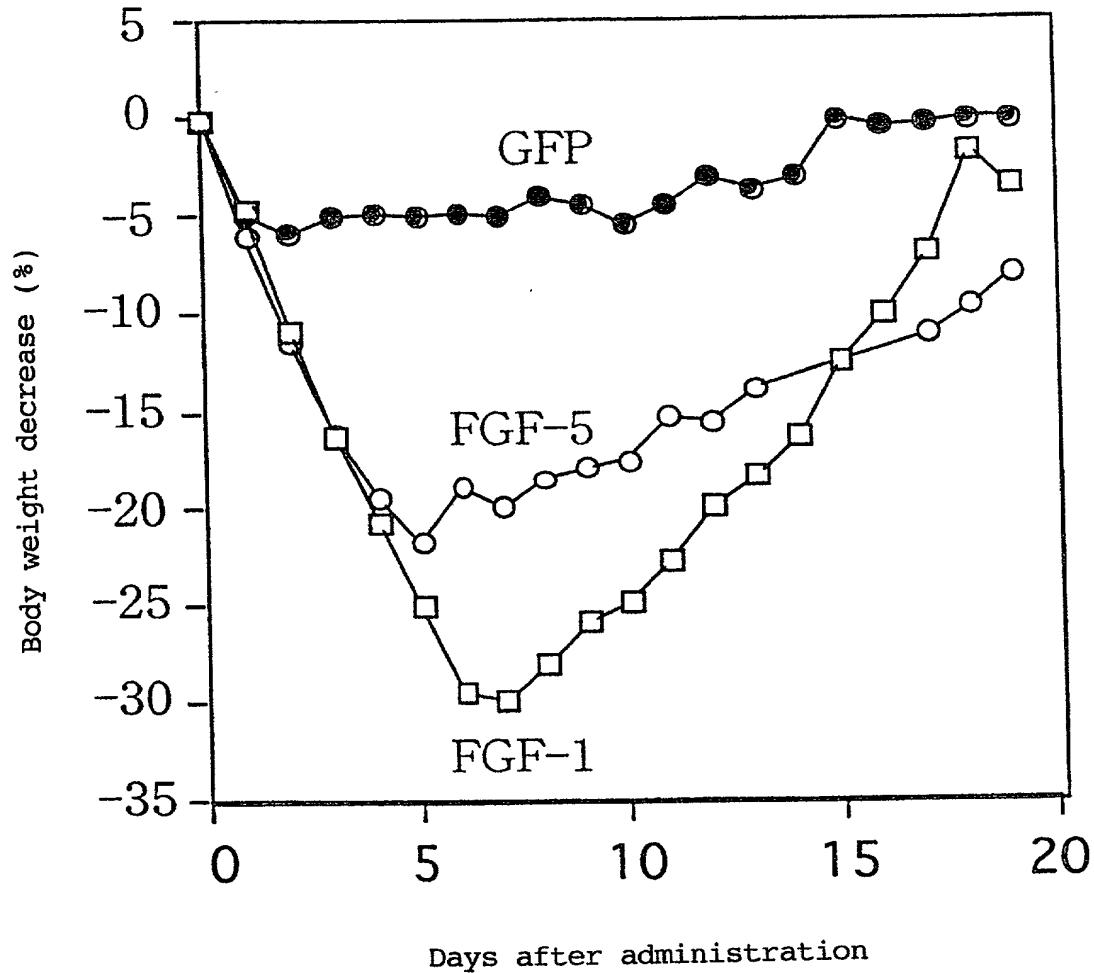
B



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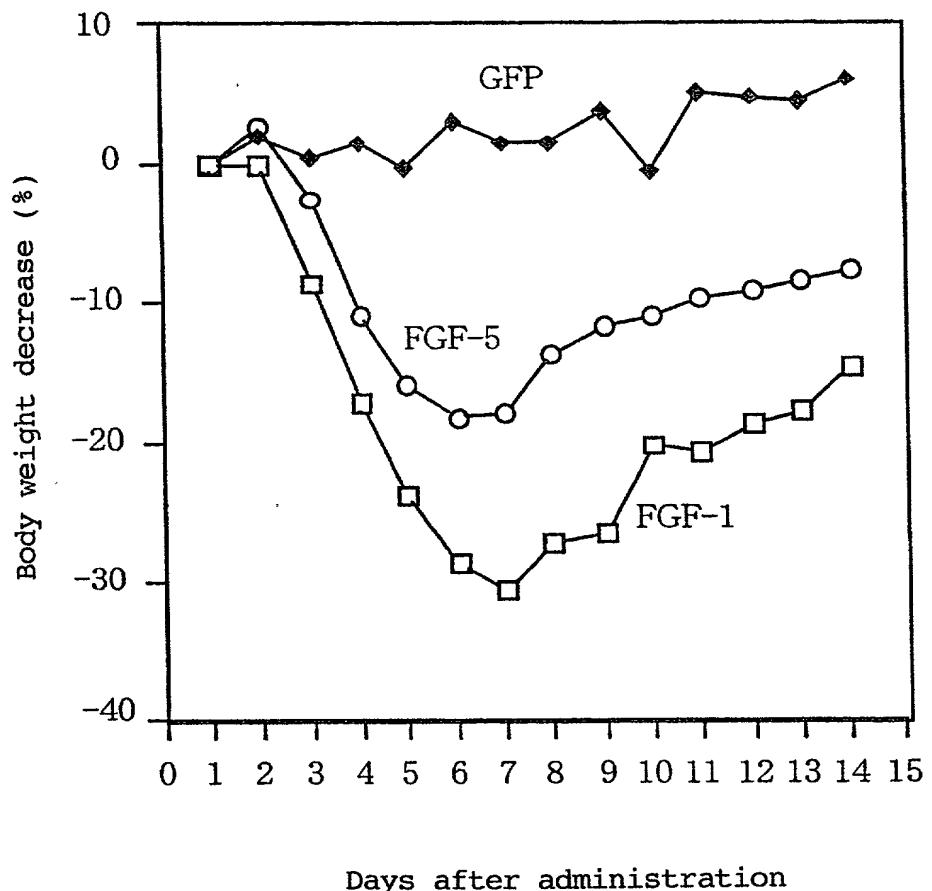
Figure 5



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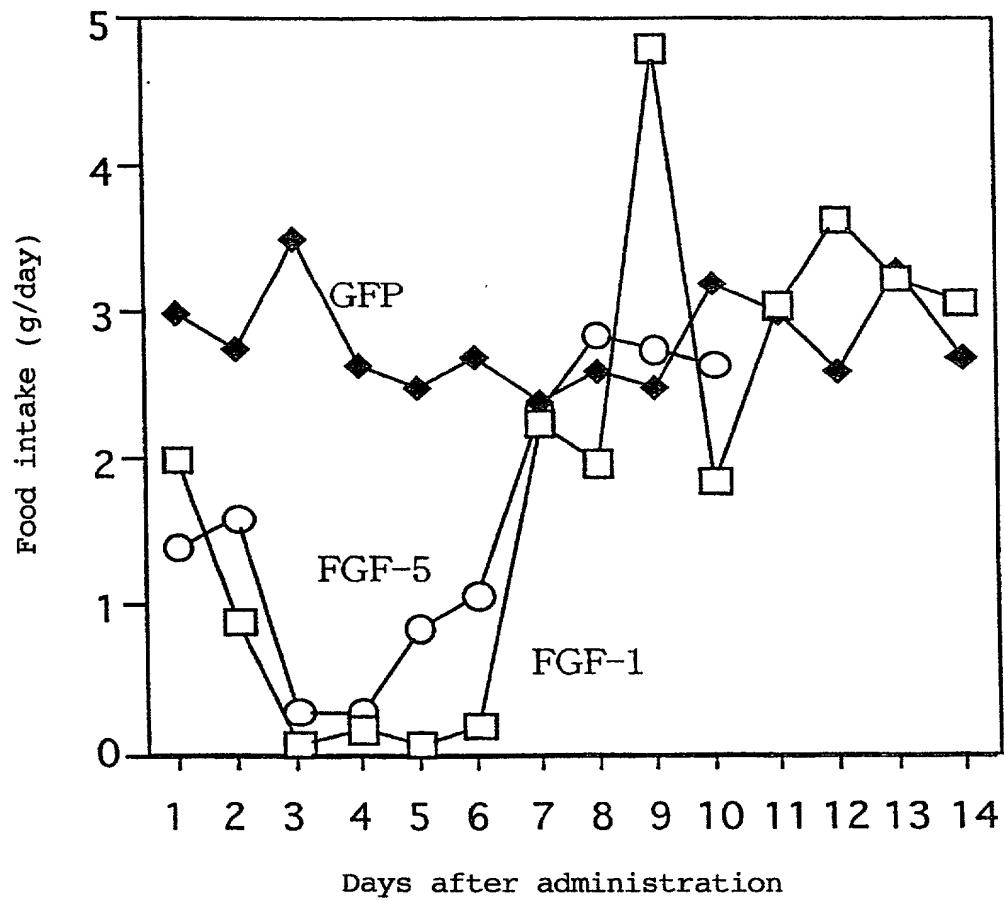
Figure 6



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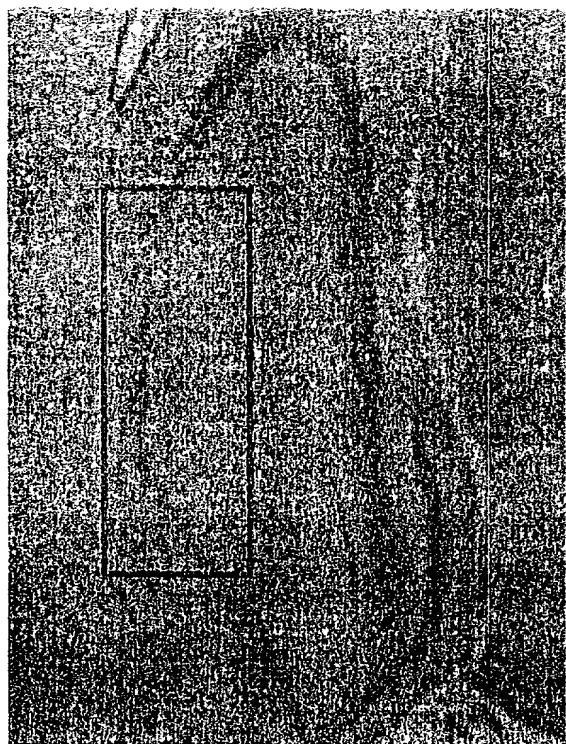
Figure 7



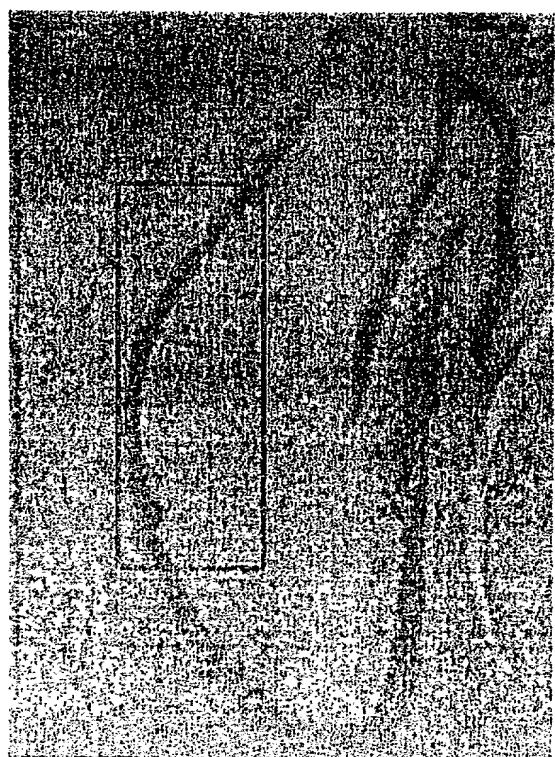
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Figure 8



Ischemia (+)



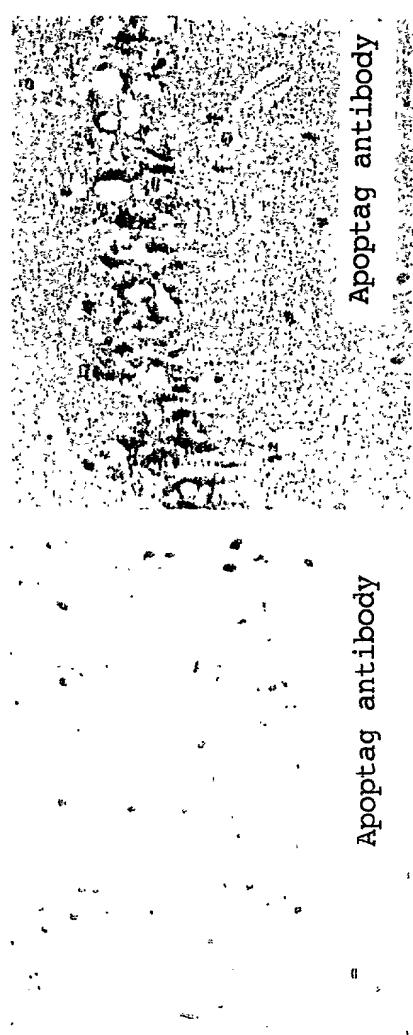
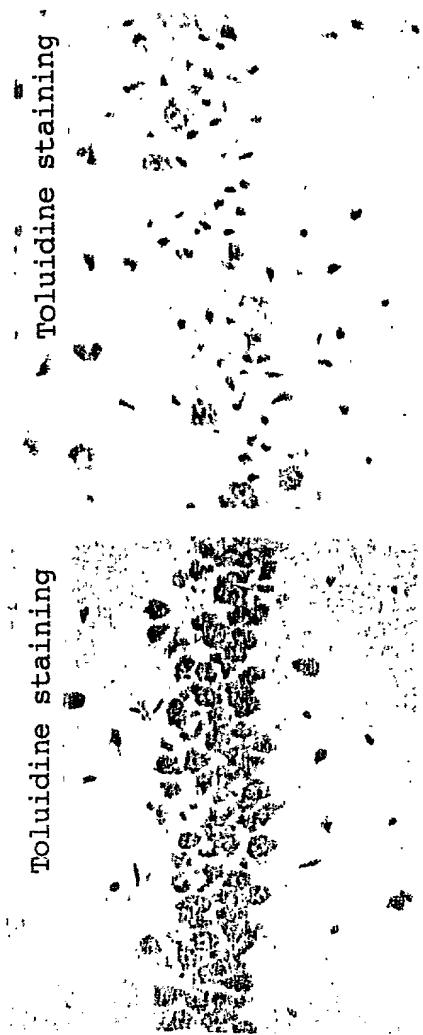
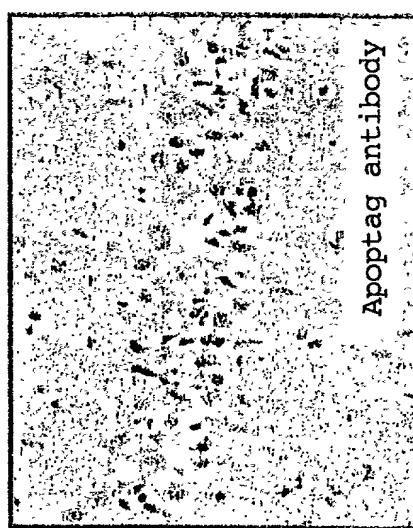
Ischemia (-)

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Figure 9

Ischemia (-) (control)      Ischemia only (left)      Recombinant SeV vector + ischemia (right)



**COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION**

As a below named inventor, I hereby declare that:

**My residence, post office address and citizenship are as stated below, next to my name.**

I believe I am the original, first, and sole inventor (if only one name is listed below) or an original, first, and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled Negative-Sense RNA Virus Vector for Nerve Cell the specification of which:

x is attached hereto.  
was filed on January 3, 2001  
as United States Application Number 09/720,979  
or PCT International Application Number \_\_\_\_\_  
and was amended on \_\_\_\_\_  
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above. I do not know and do not believe that the claimed invention was ever known or used in the United States of America before my invention thereof, or patented or described in any printed publication in any country before my invention thereof or more than one year prior to this application, that the same was not in public use or on sale in the United States of America more than one year prior to this application, and that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months (for a utility patent application) or six months (for a design patent application) prior to this application.

I acknowledge the duty to disclose all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d), of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

| Prior Foreign Application(s)          |  | Priority<br>Claimed                                 |
|---------------------------------------|--|---|
| <u>10/204333</u><br>(Number)          | <u>Japan</u><br>(Country)              | <u>03-July-1998</u><br>(Day/Month/Year Filed)       |
| <u>PCT/JP99/03552</u><br>(Number)     | <u>WO (PCT)</u><br>(Country)           | <u>01-July-1999</u><br>(Day/Month/Year Filed)       |
| <u>                  </u><br>(Number) | <u>                  </u><br>(Country) | <u>                  </u><br>(Day/Month/Year Filed) |

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below

(Application Number)

**Filing Date**

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Number) Filing Date (Status – patented, pending, abandoned)

(Application Number) Filing Date (Status – patented, pending, abandoned)

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9  
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The undersigned hereby authorizes the U.S. Attorneys named herein to accept and follow instructions from undersigned's assignee, if any, and/or, if the undersigned is not a resident of the United States, the undersigned's domestic attorney, patent attorney or patent agent, as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and the undersigned. In the event of a change in the person(s) from whom instructions may be taken, the U.S. attorneys named herein will be so notified by the undersigned.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issued thereon.

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